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TITLE: A Role for MEK-Interacting Protein 1 (MP1) in Hormone Responsiveness of Estrogen Receptor-Positive Breast Cancer Cells

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A Role for MEK-Interacting Protein 1 (MP1) in Hormone Responsiveness of Estrogen Receptor-Positive Breast Cancer Cells

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**14. ABSTRACT**

The goals of this research are to test the hypothesis that the small scaffold protein MP1 is required for ER function and proliferation of ER-positive breast cancer cells, and to characterize the ER/MP1 complex. MP1 expression was inhibited in several breast cancer cell lines by transfection with MP1-targeting siRNAs. The results obtained demonstrate that MP1 is required for the survival of ER-positive MCF-7 cells, but not ER-negative MDA-MB-231 cells. This suggests that the requirement for MP1 may be specific to ER-positive cells, in which case it could provide a novel target for treatment of this class of breast tumor. To facilitate studying the ER/MP1 complex, a Flag-MP1 gene has been cloned and reconstituted into a retrovirus vector. Cells infected with this virus efficiently express the Flag-MP1 gene for at least 9 days, and stable cell lines containing this construct are currently being selected for use in characterizing a novel ER/MP1 complex.

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ER Positive Breast Cancer, ER/MP1 Interaction, Cell Prolfieration, Cell Death

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## Introduction

In the preliminary results presented in our application, we demonstrated an interaction between estrogen receptor alpha (ER) and the small scaffold protein MP1 in breast cancer cells. In addition, we showed that overexpression of MP1 via transient transfection increased both ER's transcriptional activity and MCF-7 cell proliferation in the presence of estrogen and tamoxifen. Based on these results, we proposed that MP1 serves as a scaffold protein to organize ER-containing protein complexes, and that the ER/MP1 complex is required for ER function and estrogen regulated proliferation of breast cancer cells. To test this hypothesis, and to further characterize MP1 expression in breast cancer cells, we proposed the following two aims.

**Aim 1: To test the hypothesis that MP1 is required for ER function and proliferation in human breast cancer cells.**

**Aim 2: To characterize the subcellular localization and protein composition of ER/MP1 complexes.**

Progress on these aims is reported below, organized according to the tasks listed in the approved Statement of Work.

## Body: Progress on Tasks in Approved Statement of Work.

### Task 1: Test hypothesis that MP1 expression is required for ER's transcriptional activity and proliferation of human breast cancer cells.

**Months 1-12: Establish conditions to knock down MP1 expression in MCF-7 cells. Examine effects of MP1 knock-down on ERE-Luciferase expression in MCF-7 cells. Examine effects of MP1 knockdown on proliferation in MCF-7 cells.**

As shown in Figures 1A and 1B, we have established conditions to efficiently knock down MP1 expression in MCF-7 and other breast cancer cell lines using small interfering RNA (siRNA). Our initial hypothesis was that loss of MP1 expression would inhibit both ER's transcriptional activity and estrogen stimulated proliferation in ER-positive breast cancer cells such as MCF-7. What we have observed, however, is an even more dramatic effect. As demonstrated in Figure 1C and 1D, knock down of MP1 resulted in the death of more than 90% of MCF-7 cells within 48 h. The phenotype we observed included rounding up of cells, followed by detachment from the tissue culture plates. Knock-down of MP1 has recently been reported to lead to a defect in cell spreading in fibroblasts (1). It therefore seemed possible that the initial effect of MP1 knockdown in MCF-7 cells was a loss of adhesion to the tissue culture plates, which was then followed by detachment and cell death. To test this possibility, the viability of both attached and floating cells was assessed. As shown in Figure 1D, the vast majority of both attached and detached MCF-7 cells were non-viable by 48 h, suggesting that the primary effect of MP1

knock-down is cell death, which is then followed by rounding and detachment. Extensive cell death was not observed with a control, non-targeting siRNA (Figure 1C and 1D), but was seen with a second siRNA targeting MP1 (data not shown). Thus, the effects shown in Figure 1 are not the result of off target effects. Interestingly, knock-down of MP1 did not result in the death of MDA-MB-231, an ER-negative human breast cancer cell line (Figure 1). These results suggest that MP1 expression may be required for the survival of ER-positive, but not ER-negative cell lines. As shown in Figure 2, MP1 is expressed in all of the breast cancer cell lines that we have tested, but the requirement for its expression may be specific to ER-positive cells. In the coming year, we will test additional ER-positive and ER-negative cell lines to determine if this is the case. If it is, then MP1 may provide a novel therapeutic target in ER-positive breast tumors. We will also investigate whether the loss of viability that we have detected by trypan blue staining is due to apoptotic cell death by staining MP1 or control siRNA transfected cells for annexin V, and by assaying for PARP cleavage by Western blotting.

The fact that MCF-7 cells die within 48 h of MP1 knock-down has prevented us from using the protocol that we originally proposed to measure ER's transcriptional activity and cell proliferation. However, in the course of optimizing the siRNA transfection protocol, we observed that if cell cycle progression was blocked by treatment with the antiestrogen ICI 182,780 5 h after the addition of MP1 siRNA, MP1 was efficiently knocked down, but MCF-7 cells survived for up to 72 hours (data not shown). Our tentative conclusion from this observation is that the cell death shown in Figure 1 is in some way dependent on cell cycle progression. We therefore propose to alter our original protocol in the following way in order to complete these experiments. For ER activity assays, MCF-7 cells will be transfected with MP1 or control siRNA, +/- an ERE-luciferase reporter gene, as described in our original proposal. After five hours, the transfection medium will be removed, and fresh medium containing charcoal stripped serum (CSS) + ICI will be added to arrest the cells in G0/G1. After a 24-48 h arrest, this medium will be removed and replaced with fresh medium containing CSS alone, CSS+E, or CSS+ICI. At various times (from 3-24 h) after hormone additions, cells will be harvested and analyzed for luciferase expression. They will also be observed for evidence of cell death, as described above. To measure cell proliferation, cells will be transfected with MP1 or control siRNA, arrested with ICI, and treated with CSS, CSS+E or CSS+ICI as described above. Cells will be labeled with BrdU at various times from 4-48 h after treatment, and BrdU positive cells will be detected by immunofluorescence staining. Cell cycle position will also be monitored at the various time points by staining with propidium idodide followed by flow cytometry. These experiments will allow us to determine if ER retains transcriptional activity in the absence of MP1, whether cells can progress from G1 to S phase, and at what point (and stage in the cell cycle) cell death occurs.

**Task 2: Determine subcellular localization of ER/MP1 complexes.**

**Months 1-12: Carry out cell fractionation studies to determine subcellular localization of ER/MP1 complexes in estradiol treated MCF-7 cells.**

In our application we proposed to identify the subcellular localization of endogenous ER/MP1 complexes by biochemically isolating various cellular fractions (membrane, cytosol, nucleus, etc), and then analyzing these fractions for ER/MP1 complexes by co-immunoprecipitation. We have encountered technical difficulties that have delayed completing these experiments, specifically with the use of commercial anti-MP1 antibodies for immunoprecipitation. There are several potential reasons for these difficulties, including a change in personnel and the purchase of new batches of commercial antibodies. We are continuing attempt to complete these experiments with endogenous proteins, since we believe this approach best reflects the natural situation. This involves continued optimization of the immunoprecipitation protocol, and testing antibodies from a variety of sources. However, as described below and in Figure 3, we have also established conditions to express a Flag-tagged MP1 protein in MCF-7 cells. As an alternative approach, we will carry out cell fractionation studies in cells expressing this fusion protein, and will use antibodies to the Flag epitope for the immunoprecipitation.

**Task 3: Purify ER/MP1 complexes by sequential affinity purification and examine complex components by Western blotting.**

**Months 1-12: Purify ER/MP1 complexes from E2-treated MCF-7 transfectants. Analyze proteins present in these complexes by Western blotting with antibodies recognizing total and phosphorylated forms of these proteins.**

This initial part of this aim involved constructing stable cell lines expressing Flag-tagged MP1 in order to purify ER/MP1 complexes by sequential immunoprecipitation. We previously established a system for inducible expression of genes in breast cancer cells, and have used it to study several genes (2-3). This system is called the ARGENT system, and was developed by ARIAD Pharmaceuticals ( ://www.ariad.com/wt/page/regulation_ ) (4). It takes advantage of the fact that eukaryotic transcription factors contain two functional domains, a DNA binding domain and a transcriptional activation domain. In the ARGENT system, the DNA binding and transcriptional activation domains are expressed as separate proteins that are brought together to form a functional transcription factor by a small molecule dimerizer, AP21967 (AP). It should allow for tight and specific regulation of the target gene, since the binding site for the transcription factor does not exist in the human genome. In addition, expression should be proportional to the AP concentration, allowing one to control the level of expression of the target gene. Flag epitope tagged MP1 (Flag-MP1) was subcloned into the ARGENT Target Gene Vector (TGV), and the resulting constructs were transfected into an MCF-7 derivative (MCF-7/TF) expressing the bipartite transcription factor described above. Stable transfectants were selected with hygromycin (the TGV contains a hygromycin resistance gene), and screened for Flag-MP1 expression in the presence of AP. Although the initial screening identified several clones that expressed Flag-MP1 in the presence of AP, over time this expression was lost. These results suggested that long term expression of MP1 might be toxic to cells. The use of an inducible vector should have avoided this potential problem, since Flag-MP1 should not be

expressed in the absence of AP. However, there might be some leaky expression of the transfected gene, which could be selected against during long term passage.

The TGV contains retroviral LTRs as well as a viral packaging site, and can be reconstituted into an infectious retrovirus by transfecting the plasmid DNA into a packaging cell line such as the Phoenix Ampho line (5). To improve the efficiency with which Flag-MP1 can be expressed in breast cancer cells, the TGV-Flag-MP1 construct was reconstituted into a retrovirus, and the resulting virus stock was used to infect MCF-7/TF cells. Infected cell cultures were subjected to hygromycin selection for 9 days, then treated +/- AP for 24 h. Cell lysates were prepared and analyzed for both endogenous MP1 and Flag-MP1 expression by Western blotting with an anti-MP1 antibody. As shown in the left panel of Figure 3, the Flag-MP1 gene was efficiently expressed in these assays. Surprisingly, however, expression was not dependent upon the presence of AP. We have obtained inducible expression of several other genes, including mlk3 and c-myc, in the MCF-7/TF cell line (3, and unpublished observations), making it unlikely that the problem is due to this cell line. To confirm that this was not the case, we infected a second, independent cell line containing the bipartite transcription factor with the TGV-Flag-MP1 virus stock. This cell line was derived from non-tumorigenic MCF-10A breast epithelial cells, and is termed MCF-10A/TF. As shown in the right panel of Figure 3, expression of Flag-MP1 is also constitutive in MCF10A/TF cells. We therefore conclude that something in the TGV-Flag-MP1 construct allows Flag-MP1 to be expressed in the absence of inducer, at least when it is reconstituted into a retrovirus. If this is also the case in transfected cell lines, and if long-term overexpression of Flag-MP1 is in fact toxic, it would explain the negative selection that we observed in our original Flag-MP1 transfectants. While we were not surprised that knocking down MP1 caused cell death in MCF-7 cells, we did not predict that long-term overexpression of MP1 (or Flag-MP1) would also be toxic, especially since short term overexpression led to increased ER activity and cell proliferation. However, scaffold proteins such as MP1 function to organize protein complexes to facilitate signaling and other pathways. The stoichiometry of these proteins is critical, and either too little or too much of a scaffold protein can have deleterious effects on cells. For example, in the absence of a scaffold protein a complex may not form, but an excess of a scaffold protein may lead to sequestration of some complex components, also resulting in a lack of functional complexes. If in fact long-term overexpression of MP1 is selected against in MCF-7 cells, it would reinforce our conclusion that this protein plays a critical role in these cells.

We are currently in the process of selecting clonal lines from the TGF-Flag-MP1 retrovirus infected cultures to screen for cell lines with inducible expression of Flag-MP1. We recognize that we may encounter the same problem as in the transfected lines, which was the loss of Flag-MP1 expression over time. If this occurs, we will subclone the Flag-MP1 gene into a different inducible system, such as the “Tet-On” or “Tet-Off” system from Clontech, in order to obtain inducible cell lines. MCF-7/Tet-On cells are commercially available from Clontech, making this approach very feasible.

While continuing to screen for stable cell lines expressing Flag-MP1, we will also initiate experiments to purify ER/MP1 complexes using cells infected with the TGF-Flag-MP1 virus. As shown in Figure 3, this protein is efficiently expressed for up to 9 days after infection, and the level of expression of Flag-MP1 is similar to the endogenous protein. After 9 days of selection in hygromycin most non-infected cells will be dead. Thus, the cells remaining should represent a pool of cells expressing moderate levels of Flag-MP1, rather than a pool where most cells contain no Flag-MP1 and others contain very high levels of the protein. These pools of infected cells will be used to perform sequential affinity purifications as described in our original application, and will also be used for the cell fractionation studies described under Task 2 above.

**Task 4: Identify novel components of ER/MP1 complexes by mass spectrometry.**

Initiating this task is dependent on establishing conditions for purifying ER/MP1 complexes using Flag-MP1 and/or Flag-ER expressing cells. Our progress in establishing these cells is reported under Task 4.

**Key Research Accomplishments:**

- Established conditions for knocking down MP1 expression in breast cancer cell lines.
- Demonstrated that MP1 expression is required for survival of ER-positive MCF-7 cells, but not ER-negative MDA-MB231 cells.
- Constructed retroviral vector for efficient expression of Flag-MP1 in breast cancer cell lines.

**Reportable Outcomes:**

1) Poster Presentation at Era of Hope Meeting, June, 2008. “A Role for MEK-Interacting Protein 1 in Hormone Responsiveness of ER Positive Breast Cancer Cells”. Susan E. Conrad and Mihaela Marina. Michigan State University.

**Conclusion:** The results that we have obtained to date support an important role for MP1 in ER-positive breast cancer cell lines. Blocking MP1 expression using siRNA leads to cell death in ER-positive MCF-7 cells, and overexpression of the protein in these cells may also be toxic. The fact that the viability of the ER-negative cell line MDA-MB-231 was unaffected by MP1 knock-down suggests that the requirement for MP1 expression for may be specific to ER-positive breast cancer cells. If this is true, then MP1 could provide a novel target for the treatment of ER-positive breast tumors. Experiments in the coming year will test if the requirement for MP1 is specific to ER-positive cells, and will also characterize the ER/MP1 complex.

**References:**

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**Appendix/Supporting Data:** Figures 1-3.

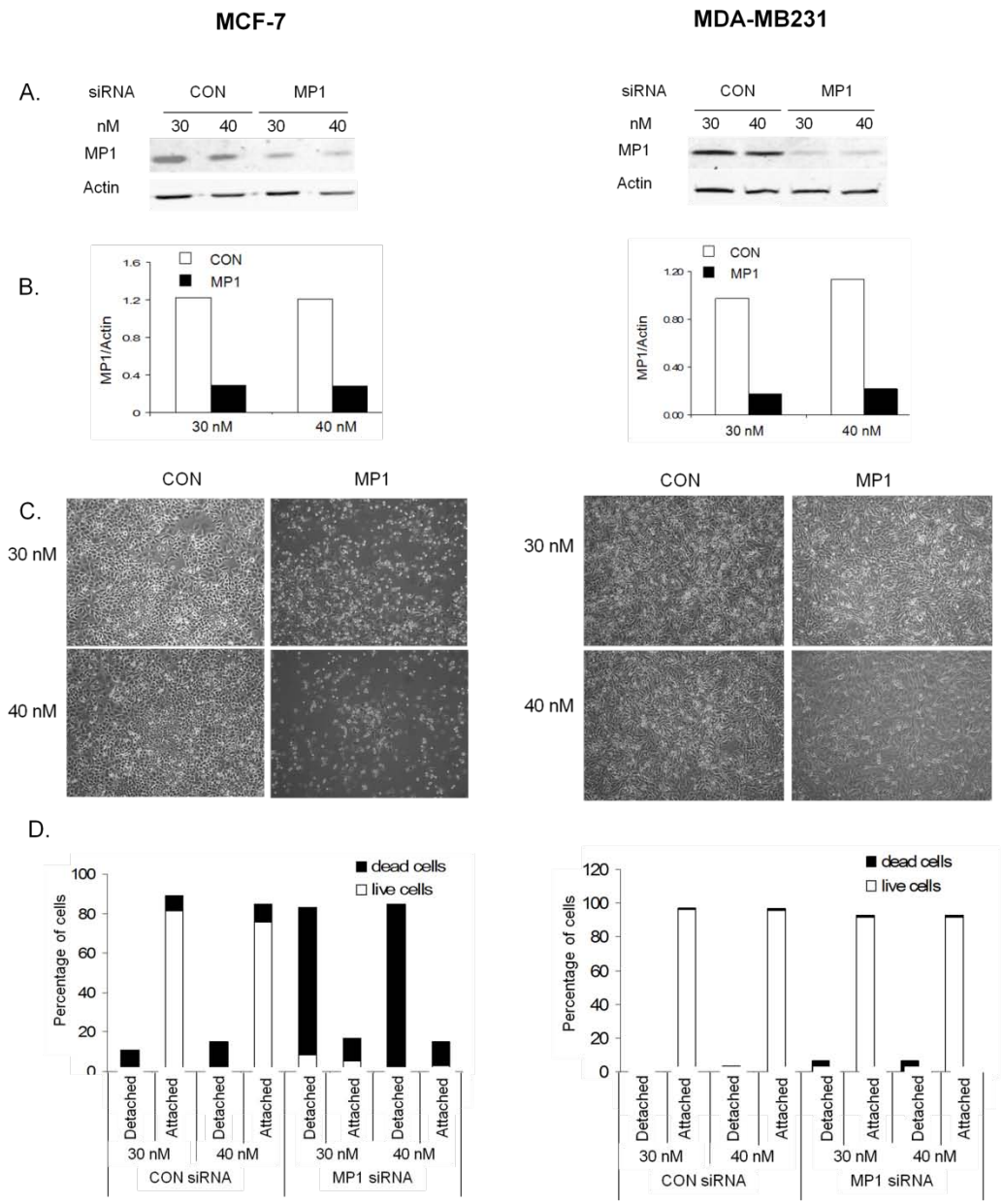


**Figure 1: Effect of MP1 knock-down on cell survival.** ER-positive MCF-7 cells or ER-negative MDA-MB-231 cells were transfected with two concentrations (30 and 40 nM) of MP1 or control siRNA. After 48 h, cells were harvested and assayed for MP1 and actin protein levels by western blotting, and for viability by trypan blue exclusion. A) Representative western blots of MP1 and actin protein levels in control and MP1 siRNA transfected cells. B) For each sample, MP1 and actin bands were quantitated using the LI-COR Odyssey System, and MP1 was normalized to actin levels. C) Photographs of representative fields of cells transfected with control and MP1 siRNA at 48 h. D) Quantitation of viable cells at 48 h. For each transfection, both attached and detached cells were harvested and stained with trypan blue to obtain viable/non-viable cell counts. The numbers shown represent the mean of two experiments.

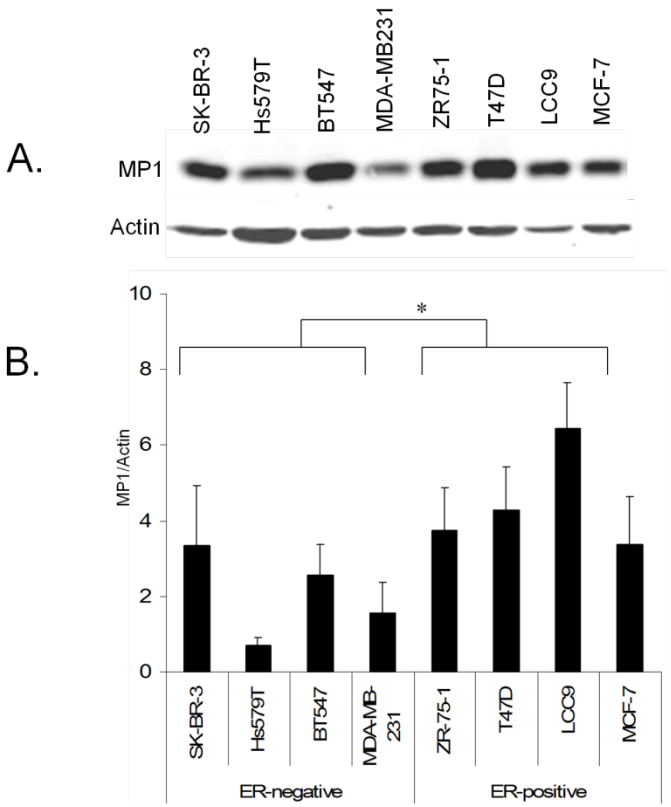


**Figure 2**. Protein extracts were prepared from a panel of ER-positive and ER-negative human breast cancer cell lines, and equal micrograms of protein from each extract were analyzed for MP1 and actin expression by western blotting. A) Photograph of representative western blot. B) For each sample, MP1 and actin bands were quantitated using the LI-COR Odyssey System, and MP1 was normalized to actin levels. The values shown are the average (+/- SD) of three independent experiments.

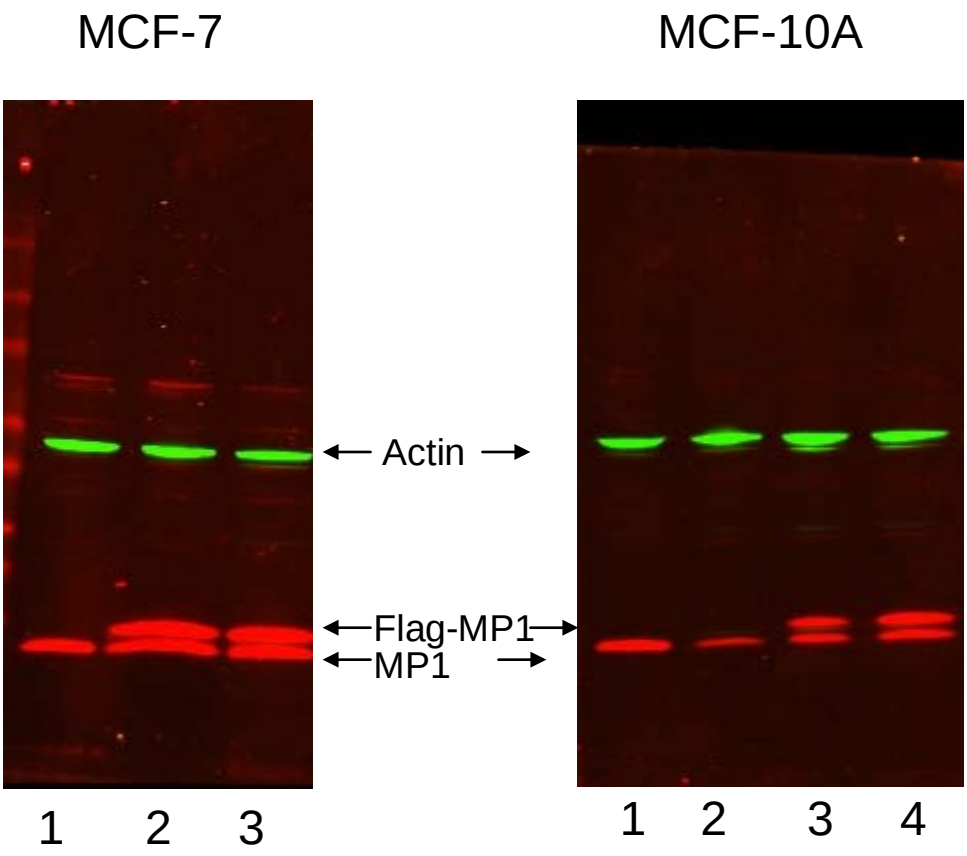


**Figure 3:** Expression of Flag-MP1 in retrovirus infected cells. MCF-7/TF cells (left panel) or MCF10A/TF cells (right panel) were infected with the TGV-Flag-MP1 vector, and subjected to hygromycin selection for 9 days. In some samples, AP was added for 24 h before harvesting. Cell extracts were prepared and analyzed for Actin and MP1 levels by Western blotting, using anti-Actin and anti-MP1 antibodies. Left Panel. Lane 1; Uninfected MCF-7/TF cell control. Lane 2; MCF-7/TF cells infected with Flag-MP1 virus, -AP. Lane 3; MCF-7/TF cells infected with Flag-MP1 virus, +AP. Right Panel. Lane 1; Uninfected MCF10A/TF cell control. Lane 2; MCF10A/TF cells infected with TGV vector. Lane 3; MCF10A/TF cells infected with Flag-MP1 virus, -AP. Lane 4; MCF10A/TF cells infected with Flag-MP1 virus, +AP.